

Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for the Evaluation of
Salmonella pullorum Antigens

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Supplemental Assay Method for the Evaluation of *Salmonella pullorum* Antigens

Table of Contents

1. Introduction
 - 1.1 Background
 - 1.2 Keywords
2. Materials
 - 2.1 Equipment/instrumentation
 - 2.2 Reagents/supplies
3. Preparation for the test
 - 3.1 Personnel qualifications/training
 - 3.2 Preparation of equipment/instrumentation
 - 3.3 Preparation of reagents/control procedures
 - 3.4 Preparation of the samples
4. Performance of the test
 - 4.1 Formalin content
 - 4.2 Hydrogen ion concentration
 - 4.3 Density
 - 4.4 Sensitivity
 - 4.5 Homogeneity
5. Interpretation of the test results
 - 5.1 Formalin content calculation
 - 5.2 Hydrogen ion concentration determination
 - 5.3 Density calculation
 - 5.4 Sensitivity determination
 - 5.5 Homogeneity determination
6. Report of test results
 - 6.1 Formalin content
 - 6.2 Hydrogen ion concentration
 - 6.3 Density
 - 6.4 Sensitivity
 - 6.5 Homogeneity

Supplemental Assay Method for the Evaluation of *Salmonella pullorum* Antigens

7. References
8. Summary of revisions
9. Appendices

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Supplemental Assay Method for the Evaluation of *Salmonella pullorum* Antigens

1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) describes how *Salmonella pullorum* antigens are analyzed by using the following procedures: a percent concentration of formalin by spectrophotometric determination; hydrogen ion concentration by a pH meter; bacterial density by spectrophotometric determination; sensitivity by a rapid agglutination test; and homogeneity by microscopic examination. These procedures are applicable to *S. pullorum* stained K polyvalent antigens, as required in the Code of Federal Regulations, Title 9 (9 CFR), Part 113.407.

1.2 Keywords

Salmonella pullorum, formalin, pH, density, sensitivity, homogeneity

2. Materials

2.1 Equipment/instrumentation

- 2.1.1 Spectrophotometer or colorimeter
- 2.1.2 Micropipettors
- 2.1.3 Pipette tips
- 2.1.4 Sterile pipettes
- 2.1.5 pH meter
- 2.1.6 Graduated cylinder, 100 ml, with ground glass stopper
- 2.1.7 Timers or stop clocks with 1 sec subdivisions

Supplemental Assay Method for the Evaluation of *Salmonella pullorum* Antigens

2.1.8 Minnesota testing box containing a glass plate with perpendicular etched lines forming 3 cm x 3 cm squares

2.1.9 Aluminum mixing device designed for use with the slide agglutination test

2.1.10 Glass slides and coverslips

2.1.11 Light microscope

2.1.12 Vortex mixer

2.2 Reagents/supplies

2.2.1 Reagent A (or acid solution)--dilute hydrochloric acid (**Section 8.3**), 2.5% (v/v)

2.2.2 Reagent B (or formalin solution)--(National Veterinary Services Laboratories [NVSL] Media #30038), standard formalin, 0.2 ml of formaldehyde (U.S.P.), diluted to 100 ml with purified water

2.2.3 Reagent C (or indicator solution)--fuchsin-sulfurous acid (**Section 8.2**)

2.2.4 pH buffer solutions

2.2.5 5% aqueous hydrochloric (HCl) acid (**Section 8.3**)

2.2.6 Purified water (H₂O)

2.2.7 *S. pullorum* serums (prepared according to the current version of STPRO0003)--A total of at least 12 positive serums shall be used. This shall include a diluted and an undiluted of each of the 3 definitely positive (high titer) serums--regular (R), intermediate (I), and variant (V) and a diluted and an undiluted of each of the 3 weakly positive (low titer) serums--R, I, and V. In addition, 6 negative serums shall also be used. The 3 definitely positive and the 3 weakly

Supplemental Assay Method for the Evaluation of *Salmonella pullorum* Antigens

positive serums are diluted with negative chicken serum so that a comparison of the sensitivity between the product and the reference plate antigens, within the specified time limit of 2 min, can be made. The dilutions may vary with the *S. pullorum* strains and serum production lots. Six *S. pullorum*-negative serums are used to detect nonspecific reactions. The 18 positive and negative serums are produced by the Cytology and Sterility (CS) section at the Center for Veterinary Biologics-Laboratory (CVB-L).

2.2.8 *S. pullorum* stained K antigen reference (prepared according to the current version of STPRO0002)--to compare product on test to a known reference antigen.

2.2.9 McFarland standard No. 1 (prepared according to the current version of STPRO0002)--A turbidimetric McFarland standard No. 1 cell suspension is produced by the CS section for use in this procedure.

2.2.10 Immersion oil

2.2.11 20% HCl acid solution--NVSL Media #30036 (Section 8.3)

3. Preparation for the test

3.1 Personnel qualifications/training

The personnel must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques, as well as preparation, proper handling, and disposal of biological agents, reagents, tissue culture samples, and chemicals. The personnel must also have knowledge of safe operating procedures and policies and Quality Assurance (QA) guidelines of the CVB-L or equivalent, as well as training in the operation of the necessary laboratory equipment listed in **Section 2.1**.

Supplemental Assay Method for the Evaluation of *Salmonella pullorum* Antigens

3.2 Preparation of equipment/instrumentation

Turn on the spectrophotometer to 'warm up' for at least 10 min.

3.3 Preparation of reagents/control procedures

Reagents are prepared by the NVSL Media and CVB-L CS sections. Allow samples, serums, and reagents to warm to room temperature before conducting the tests.

3.4 Preparation of the samples

Samples are *S. pullorum* stained K polyvalent antigens with product codes 5206.00 or 5207.00. Samples are acquired from the Biological Materials Processing Section (BMPS) according to the current version of STSOP0001.

4. Performance of the test

4.1 Formalin content

4.1.1 For each sample of the antigen to be tested, pipette 10 ml of 2.5% HCl acid solution (prepared from 20% HCl acid solution, **Section 8.1**) into test tube 'A' and 11 ml into test tube 'B.' Pipette 10 ml of 2.5% HCl acid solution into each of 7 test tubes, 1 through 7. Deliver 50 μ L of the product into each test tube, 'A' and 'B,' using a pipette. Set test tube 'B' aside. Prepare a standard curve of known concentrations using test tubes 1 through 7 with 10 ml of 2.5% HCl acid solution in each. Add 2% formalin to each of the 7 test tubes as follows:

Tube #:	1	2	3	4	5	6	7
ml:	0.15	0.20	0.225	0.25	0.275	0.30	0.35

Mix well. Allow the tubes to sit for 1 hr to decolorize the dye in tubes 'A' and 'B.'

Supplemental Assay Method for the Evaluation of *Salmonella pullorum* Antigens

4.1.2 Add 1 ml of Reagent C, fuchsin-sulfurous acid, to test tube 'A' and test tubes 1 through 7. Tube 'B' is a turbidity blank; do not add fuchsin-sulfurous acid to this. Mix well and allow to sit for 10 min. Shake all tubes well before reading immediately after 10 min incubation. Zero the spectrophotometer using a wavelength of 570 nanometers (nm), and adjust the transmittance to 100% with solution in tube 'B' 5 min before the incubation period is finished. Record the transmittances of the solutions in tubes 'A' and 1 through 7 on STFRM4070.

4.2 Hydrogen ion concentration

4.2.1 Standardize the pH meter with pH buffer solutions 4 and 7 (described in the current version of GDOCSOP0009). Dispense 2 ml of antigen into a screw-capped culture tube. Insert the pH meter electrode into the tube containing the antigen. Record results on worksheet STFRM4070.

4.3 Density

4.3.1 Place 49 ml of a 5% (by volume) aqueous hydrochloric acid (reagent grade) solution (**Section 8.3**) in a 100-ml graduated cylinder with a ground glass stopper. Shake or vortex the antigen before sampling. Using a pipettman, add 1 ml of the well-shaken antigen to the hydrochloric acid solution to make a 1-in-50 dilution of the antigen. Shake the mixture and let stand at least 2 hr.

4.3.2 Zero the spectrophotometer; using a wave length of 420 nm, adjust the transmittance to 100% with H₂O, then determine the bacterial density of the Turbidimetric Standard Cell Suspension (McFarland standard No. 1, **Section 2.2.9**). Determine the bacterial density of the diluted antigen sample by adding increments of H₂O until it equals the density of McFarland standard No. 1. (This is accomplished by adding 5 ml of H₂O to the graduated cylinder containing the sample, mixing, removing a sample to check

Supplemental Assay Method for the Evaluation of *Salmonella pullorum* Antigens

turbidity with the spectrophotometer, returning the sample to the graduated cylinder, adding more water, mixing, then repeating the process.) Smaller increments may be used as equivalence is approached. Record the total amount of H₂O (50 + added credence) on the worksheet (STFRM4070).

4.4 Sensitivity

4.4.1 Perform the rapid plate agglutination test for stained K antigens as follows: Warm test antigen, reference antigen, and serums to room temperature (22° to 26°C). Warm Minnesota box testing plate to approximately 37°C. Mix test antigen and reference antigen by shaking for 1 min by a wrist-action motion or by vortex mixing. Using a pipettman, place 20 µL of each serum within each of 2 separate squares on the plate of the Minnesota testing box. Add 1 drop of test antigen to 1 of the squares containing serum with the dropper that is provided by the manufacturer with each antigen vial. Add 1 drop of reference antigen to the duplicate serum. Mix 4 squares simultaneously with the aluminum mixing device being moved in a clockwise manner. Turn a stop clock on immediately following mixing. Pick up and gently tip the glass plate in a circular motion to swirl the liquid. Run each agglutination for 2 min; anything that does not agglutinate in 2 min is negative for agglutination. Record the results for both the reference and product antigen for all serums used as the number of seconds elapsing until a positive agglutination is observed. Record those results on the worksheet (STFRM4070).

4.5 Homogeneity

4.5.1 Prepare a wet mount by placing a drop of antigen on a microscope slide, adding a cover slip, and examining under the oil immersion objective. Record the observation of homogeneous or heterogeneous on the worksheet (STFRM4070).

Supplemental Assay Method for the Evaluation of *Salmonella pullorum* Antigens

5. Interpretation of the test results

5.1 Formalin content calculation

Record the transmittance for tubes 'A' and 2 through 6 on the worksheet, STFRM4070. Determine the tube in the standard curve that has a transmittance similar to the product (tube 'A') and determine the formalin equivalent (%) from the following chart:

Tube #:	1	2	3	4	5	6	7
Formalin (%):	0.7	0.8	0.9	1.0	1.1	1.2	1.4

For example, if product (tube 'A') transmittance is between the transmittances of formalin standard tubes No. 3 and No. 4, then the product sample has approximately 0.95% formalin. The formalin content of *S. pullorum* stained K antigens must be $1.0 \pm 0.2\%$ for a satisfactory (SAT) result. Record the results on the worksheet, and report out under CS test code 407-CH0.

5.2 Hydrogen ion concentration determination

Stained K antigens must have a pH of 4.6 ± 0.4 for a SAT result. Record the results on the worksheet and report out under CS test code 407-CH3.

5.3 Density calculation

The density, which is expressed as a multiple of McFarland No. 1 standard, can be defined as the reciprocal of the mls of water required to effect a turbidity equivalent to the McFarland standard.

Example:

$(50 + n)$ = number of ml required to equal turbidity of McFarland No. 1 (n = number of additional ml of water that need to be added). If n is 25, then the bacterial density is 75 times McFarland standard No. 1. The bacterial density must be 80 ± 15 times the McFarland standard No. 1 cell

Supplemental Assay Method for the Evaluation of *Salmonella pullorum* Antigens

suspension for a SAT result. Record results on the worksheet (STFRM4070).

5.4 Sensitivity determination

A positive result means definite clumping (agglutination) with clearing of fluid. A negative result means the serum and antigen mixture remains uniformly turbid. Reactions are read within 2 min. If agglutination is found in at least 5 of the positive serums, the test is satisfactory (SAT). In the event of an UNSAT test, at least 3 additional definitely positive (high titer) and 3 additional weakly positive (low titer) serums shall be used. If agglutination is found in 5 or 6 of the above serums, the test is concluded as being SAT. If agglutination is found with any of the negative serums within the time limit of 120 sec, repeat the test once, but if agglutination is found, the serial results are concluded as being UNSAT. Record results on the worksheet and report out under CS test code 407-PT1.

5.5 Homogeneity determination

Examine the test antigen for autoagglutination or an unusual appearance, such as the presence of flakes or specks when examined microscopically. The observation of autoagglutination or an unusual appearance would make the product serial UNSAT for homogeneity. Record results as homogeneous or heterogeneous on the test worksheet to denote a SAT or UNSAT conclusion, respectively, and report out under CS test code 407-PU1.

6. Report of test results

6.1 Formalin content

After determining the formalin content for the product tested, record on the log book worksheet (STFRM4070) and the computer worksheet, along with the final conclusion for the status of the product. Enter this information into the computer under CS test code 407-CH0.

Supplemental Assay Method for the Evaluation of *Salmonella pullorum* Antigens

6.2 Hydrogen ion concentration

Record the pH of the product tested, along with the final test conclusion, on the log book worksheet (STFRM4070) and the computer worksheet. Enter this information into the computer under CS test code 407-CH3.

6.3 Density

Record the density results on the log book worksheet (STFRM4070) and the computer worksheet, along with the final conclusion, for the status of the product tested. Enter this information into the computer under CS test code 407-PT0.

6.4 Sensitivity

Record the number of negative serums that were negative and the number of positive serums that were positive on the log book worksheet (STFRM4070) and the computer worksheet, along with the final conclusion for the status of the product tested. Enter this information into the computer under CS test code 407-PT1.

6.5 Homogeneity

Record the result as homogeneous or heterogeneous on the log book worksheet (STFRM4070) and the computer worksheet, along with the final conclusion for the status of the product tested. Enter this information into the computer under CS test code 407-PU1.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.407,
U.S. Government Printing Office, Washington, DC, 1999.

Supplemental Assay Method for the Evaluation of *Salmonella pullorum* Antigens

8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. The following is a list of significant changes made from the superseded protocol:

8.1 The sensitivity testing of *S. pullorum* antigens, Part B. of the April 11, 1986, SAM 602, has been divided into the various sections of this SAM. The Materials section (1.a.) is explained in **Section 2.1** (Equipment/instrumentation); the antisera section (1.b.) is explained in **Section 2.2** (Reagents/supplies); the Methods section (2.a.) is explained in **Section 4.4** (Sensitivity); and the Interpretation section (2.b.) is explained in **Section 5.4** (Sensitivity determination).

8.2 The density testing of the *S. pullorum* antigens, Part C. of the April 11, 1986, SAM 602, has been divided into various sections of this SAM. The Materials and Methods section (C.1.) has been divided and is explained in **Sections 2.1 and 2.2** (Materials) and **Section 4.3** (Performance of the test). The Calculations section (C.2.) is explained in **Section 5.3** (Density calculation).

8.3 The test for preservative concentration of formalin in *S. pullorum* antigens has been changed from the test described in SAM 501 to a test which is similar to the method described in the May 5, 1997, version of TCSAM0510. This formalin test is described in various sections of this SAM; the equipment and reagents are described in **Sections 2 and 9**, the performance of the test in **Section 4.1**, and the formalin content calculation in **Section 5.1**.

8.4 The test for homogeneity is unchanged in this SAM, with the performance of the test being found in **Section 4.5** and the homogeneity determination in **Section 5.5**.

8.5 The test for hydrogen ion concentration is unchanged in the SAM, with the performance of the test being found in **Section 4.2** and the hydrogen ion determination in **Section 5.2**.

Supplemental Assay Method for the Evaluation of *Salmonella pullorum* Antigens

9. Appendices--Media Formulations

9.1 NVSL Media Formulation #30038

Formaldehyde-salt solution

NaCl	8.5	g
Formaldehyde	10.0	ml
Q H ₂ O up to	1000.0	ml

Dissolve the NaCl in the Q H₂O and dispense into requested container(s). Cover and autoclave 30 min to sterilize. When solution has cooled, aseptically add the formaldehyde.

9.2 NVSL Media Formulation #30045

Fuchsin-sulfurous acid (Reagent C)

Basic fuchsin	0.1	g
Dissolve in H ₂ O	90.0	ml
Add sodium sulfite	1.0	g
When dissolved add HCl	20.0	ml

9.3 NVSL Media Formulation #30036

20% Hydrochloric acid

HCl	20.0	ml
Q H ₂ O	80.0	ml

Add the HCl to the Q H₂O. Mix and dispense into requested container(s).

To prepare a 5% working solution HCl, place 25 ml of the 20% hydrochloric acid solution in 75 ml of Q H₂O.

To prepare a 2.5% working solution of HCl, place 12.5 ml of the 20% hydrochloric acid solution in 87.5 ml of H₂O.